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SIMPLE SYNTHETIC MEDIA FOR THE GROWTH OF *B. COLI* AND FOR ITS ISOLATION FROM WATER.*

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THE purpose of this research was first, to prepare a simple synthetic culture medium for the growth of *B. coli*, and second, to find a simple synthetic medium to be used in water analysis, which would favor the growth of *B. coli* and would inhibit the growth of the other organisms commonly present in water.

The advantages of a synthetic medium are plain. As its chemical composition is exactly known it can be easily and quickly prepared, and can always be duplicated. Results obtained by its use are always uniform and comparable. By the use of such a medium some light may be thrown on bacterial metabolism.

I. SIMPLE SYNTHETIC MEDIA FOR THE GROWTH OF *B. coli*.

Some experimental work has been done already with synthetic culture media for the growth of *B. coli*. Galimard and Lacomme[1] give the chemical composition of several media on which *B. coli* and certain other organisms grow well. They worked with amido acids as the bases of their media. One of their media had the following composition:

Leucin	7.5 gr.
Alanin	2.0 gr.
Tyrosin	traces
Glycerin	15 gr.
Sodium chloride	0.5 gr.
Magnesium sulphate	0.05 gr.
Calcium glycerophosphate	0.2 gr.
Sodium carbonate	to neutralize
Distilled water	1,000 c.c.

The following organisms were found to grow on this medium:

B. pyocyaneus
B. prodigiosus
B. coli R[2]
Pneumobacillus of Friedlander
B. tetragenus
Staphylococcus aureus
Sarcina superba

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* Received for publication October 29, 1908.

[1] *Jour. de physiol. et path. générale*, 1907, 9, p. 481.

B. coli was also grown on several other media similar to the above but containing other amido acids such as glycocoll, aspartic acid, etc.

MacConkey[1] showed that *B. coli* and other intestinal bacteria grow well on an agar medium containing 0.5 per cent of sodium taurocholate, while the common bacteria are to a great extent inhibited.

Jackson[2] states that the bile salts, when added to a Standard agar medium, exert a strong restraining action on most species of bacteria which grow at blood heat. This restraining action is selective, it favors the growth of *B. coli*, retards the growth of certain streptococci, and actually kills off the majority of species which grow at 37° C. Jackson says that the effect must be due to the cholic acid radical which is common to both salts.

Both MacConkey's and Jackson's media have a very complex chemical composition and a variable one. Galimard and Lacomme's medium has the advantage of having a fixed chemical composition but is still too complex to be of practical value in water analysis. If their medium could be simplified and if the inhibitive property of Jackson's medium could be given to it we would have a medium of practical value.

As *B. coli* was known to grow on several synthetic media containing amido compounds the following was tried:[3]

Sodium chloride	0.5 gr.
Disodium phosphate	0.2 gr.
Ammonium lactate	0.6 gr.
Asparagin	0.4 gr.
Distilled water	100 c.c.
Sodium carbonate	to neutralize

B. coli freshly isolated from feces grew very well on this medium. After 12 hours at 37° C. there was a strong turbidity in the inoculated tubes; after 24 hours there was a heavy sediment at the bottom of the tubes and a pellicle at the surface.

Ammonium lactate was then omitted from the medium and the following tried:

[1] *Thompson Yates Laboratories Rep.*, 1900, 3, p. 41; 1901, 3, p. 151; MacConkey, A., and Hill, Chas. *ibid.*, 1901, 4, p. 151.

[2] *Biological Studies by the Pupils of William Thompson Sedgwick*, Boston, 1906, p. 292.

[3] In all cases throughout this work C. P. chemicals and water distilled in glass were used.

Sodium chloride	0.5 gr.
Disodium phosphate	0.2 gr.
Asparagin	1 gr.
Distilled water	100 c.c.
Sodium carbonate	to neutralize

B. coli also grew well on this medium. This medium contains but one organic compound, the other substances are all inorganic salts.

The next experiment was to determine whether *B. coli* can grow on amido compounds alone. The results are given in Table 1.

TABLE 1.

Number of Medium	Composition of Medium		Growth of *B. coli* at 37° C.	
			In 24 Hours	In 48 Hours
1	Glycocoll Distilled water	1 gr. 100 c.c.	None	None
2	Glycocoll Sodium chloride Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
3	Glycocoll Potassium chloride Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
4	Asparagin Distilled water	1 gr. 100 c.c.	None	None
5	Urea Distilled water	1 gr. 100 c.c.	None	None

All made neutral to phenolphthalein with NaOH.

Under the above conditions *B. coli* does not grow on glycocoll, asparagin, or urea alone. These experiments are controls on the purity of our salts and of our distilled water and their freedom from the salts which we shall find later are required for the growth of *B. coli.*

The next experiments were tried to show what inorganic salts *B. coli* requires in addition to asparagin. The latter was selected as the organic base of the media not only because it was less expensive than the others but also because further experiments showed it to be the most favorable. The more common soluble inorganic salts were tried. The results are given in Table 2.

The experiments in Table 2 show that *B. coli* grows on a combination of asparagin with either sodium or ammonium nitrate or sodium or ammonium phosphate but grows much better on the phosphates than on the nitrates.

TABLE 2.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM		GROWTH OF *B. coli* AT 37° C.	
			In 24 Hours	In 48 Hours
6	Asparagin NaCl Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
7	Asparagin KCl Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
8	Asparagin NH_4Cl Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
9	Asparagin $CaCl_2$ Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
10	Asparagin $MgCl_2$ Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
11	Asparagin Na_2SO_4 Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
12	Asparagin K_2SO_4 Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
13	Asparagin $(NH_4)_2SO_4$ Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
14	Asparagin $NaNO_3$ Distilled water	1 gr. 0.2 gr. 100 c.c.	Slight	Slight
15	Asparagin KNO_3 Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
16	Asparagin NH_4NO_3 Distilled water	1 gr. 0.2 gr. 100 c.c.	Very slight	Slight
17	Asparagin Na_2HPO_4 Distilled water	1 gr. 0.2 gr. 100 c.c.	Good	Very good
18	Asparagin K_2HPO_4 Distilled water	1 gr. 0.2 gr. 100 c.c.	None	None
19	Asparagin $(NH_4)_2HPO_4$ Distilled water	1 gr. 0.2 gr. 100 c.c.	Good	Very good

All made neutral to phenolphthalein with NaOH.

The next step was to determine whether the concentration of these salts has any influence on the growth and whether the combination of two salts would be an improvement.

The following table (Table 3) gives the results there enumerated. As far as could be noticed the increase in the concentration of the salts or the combination of two salts does not improve the growth.

TABLE 3.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM		GROWTH OF *B. coli* AT 37° C.	
			In 24 Hours	In 48 Hours
20	Asparagin $NaNO_3$ Distilled water	1 gr. 0.2 gr. 100 c.c.	Very slight	Slight
21	Asparagin $NaNO_3$ Distilled water	1 gr. 0.4 gr. 100 c.c.	None	No better than on 20
22	Asparagin NH_4NO_3 Distilled water	1 gr. 0.2 gr. 100 c.c.	None	Very slight
23	Asparagin NH_4NO_3 Distilled water	1 gr. 0.4 gr. 100 c.c.	None	No better than on 22
24	Asparagin Na_2HPO_4 Distilled water	1 gr. 0.2 gr. 100 c.c.	Good	Very good
25	Asparagin Na_2HPO_4 Distilled water	1 gr. 0.4 gr. 100 c.c.	Good; same as on 24	Very good; same as on 24
26	Asparagin $(NH_4)_2HPO_4$ Distilled water	1 gr. 0.2 gr. 100 c.c.	Good	Very good
27	Asparagin $(NH_4)_2HPO_4$ Distilled water	1 gr. 0.4 gr. 100 c.c.	Good; same as on 26	Very good; same as on 26
28	Asparagin $NaNo_3$ Na_2HPO_4	1 gr. 0.2 gr. 0.2 gr.	Good; same as on 24	Very good; same as on 24
29	Asparagin NH_4NO_3 $(NH_4)_2HPO_4$ Distilled water	1 gr. 0.2 gr. 0.2 gr. 100 c.c.	Good; same as on 26	Very good; same as on 26
30	Asparagin $NaNO_3$ $(NH_4)_2HPO_4$ Distilled water	1 gr. 0.2 gr. 0.2 gr. 100 c.c.	Good	Good
31	Asparagin $NaNO_3$ NH_4NO_3 Distilled water	1 gr. 0.2 gr. 0.2 gr. 100 c.c.	Slight	Good
32	Asparagin Na_2HPO_4 $(NH_4)_2HPO_4$ Distilled water	1 gr. 0.2 gr. 0.2 gr. 100 c.c.	Good	Good

All made neutral to phenolphthalein with NaOH.

We may decide then that either one of the two following media may be considered favorable for the growth of *B. coli* at 37° C. A good growth is obtained even in the first 24 hours.

MEDIUM 1		MEDIUM 2	
Asparagin	1 gr.	Asparagin	1 gr.
Na_2HPO_4	0.2 gr.	$(NH_4)_2HPO_4$	0.2 gr.
Distilled water	100 c.c.	Distilled water	100 c.c.

Made neutral to phenolphthalein with NaOH.

2. THE USE OF THE ABOVE MEDIA FOR THE ISOLATION OF *B. coli* FROM WATER.

A. *Lactose-asparagin agar.*—Inasmuch as in the routine of water analysis a solid medium is required we next directed our attention to the combination of the simple media described, with agar, for the purpose of isolating the colon bacillus by the plate method.

The agar was purified by cutting it into small pieces and soaking in distilled water for 24 hours, and the following medium which may be called lactose-asparagin agar was prepared:

Purified agar (3 per cent solution)		250 c.c.
Asparagin	2.5 gr.	250 c.c.
Na_2HPO_4	0.5 gr.	
Distilled water	250 c.c.	

Made neutral to phenolphthalein with NaOH and 1 per cent lactose added.

Plates were made using this lactose-asparagin agar and 1 per cent azolitmin[1] solution in the usual manner. Several samples of water slightly polluted with *B. coli* were plated with the azolitmin-lactose-asparagin agar. Numerous colonies developed in each plate after 24 hours at 37° C. Several red colonies were isolated and proved to be *B. coli.*

The above medium, judging from repeated trials, appears to be just as effective as the Standard litmus-lactose agar for the isolation of *B. coli* and besides it has the advantage of having a fixed and simple chemical composition.

B. *The addition of certain substances to the above medium for the purpose of inhibiting the growth of the bacteria in water other than B. coli.*—Jackson has proved by experiments that the bile salts (sodium taurocholate and glycocholate) favor the growth of *B. coli* and restrain the growth of other forms of water bacteria. He proved that this restrictive action was due to the cholic group of the bile salts.

Sodium glycocholate and taurocholate could be added to the above medium to restrain the growth of other bacteria. But pure bile salts are too expensive to be used on a large scale. The use of bile itself would defeat the object of these experiments as its composition is so complex and variable.

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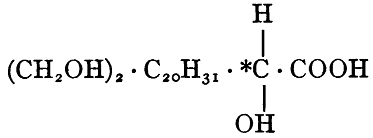
[1] There are several kinds of azolitmin on the market. Kahlbaum's C. P. azolitmin was used exclusively in these experiments. One gram was dissolved in 100 c.c. of distilled water and the solution boiled for 15 minutes. The solution which at first is slightly red turns to a blue after boiling. Prepared in this way the azolitmin solution does not need to be treated with an alkali to bring it to the right reaction. It may be used directly with any culture medium which is neutral to phenolphthalein. Other samples of azolitmin do not act in this way.

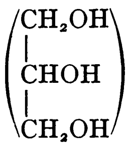
It was thought that very probably simpler and cheaper substances could be found which would have the same selective action. A consideration of the chemical structure of cholic acid led to the following: Cholic acid is



$$(CH_2OH)_2 \cdot C_{20}H_{31} \cdot \overset{\displaystyle H}{\underset{\displaystyle OH}{{}^*C}} \cdot COOH$$

It has two primary alcohol groups (CH_2OH),and one a symmetrical carbon atom indicated by a star (*). Many substances could be selected having either an asymmetrical carbon atom, or the (CHOH) group, or the (CH_2OH) group, or all of them combined. The following substances were tried:

Glycerin



$$\left(\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array}\right)$$

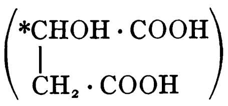
which has two primary alcohol groups and one secondary alcohol group.

Ammonium lactate

$$(CH_3 \cdot {}^*CHOH \cdot COO \cdot NH_4)$$

which has the secondary alcohol group and an asymmetrical carbon atom.

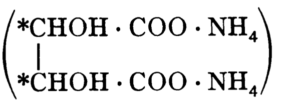
Malic acid



$$\left(\begin{array}{l} {}^*CHOH \cdot COOH \\ \quad | \\ \ CH_2 \cdot COOH \end{array}\right)$$

which has the secondary alcohol group and an asymmetrical carbon atom.

Ammonium tartrate



$$\left(\begin{array}{l} {}^*CHOH \cdot COO \cdot NH_4 \\ \quad | \\ {}^*CHOH \cdot COO \cdot NH_4 \end{array}\right)$$

which has two secondary alcohol groups and two asymmetrical carbon atoms.

Experiments were then begun to determine whether these various substances could be substituted for the asparagin with the idea that if this is possible they might serve both as a food for *B. coli* and as inhibitory agents for the other organisms.

The results are given in the following table (Table 4):

TABLE 4.

NUMBER OF MEDIUM	COMPOSITION OF MEDIUM	GROWTH OF *B. coli* AT 37° C.		
		In 24 Hours	In 48 Hours	In 72 Hours
33	Glycerin 1 gr. $(NH_4)_2HPO_4$ 0.2 gr. Distilled water 100 c.c.	Good	Very good	Very good
34	Ammonium lactate 1 gr. Na_2HPO_4 0.2 gr. Distilled water 100 c.c.	Good	Very good	Very good
35	Malic acid 1 gr. $(NH_4)_2HPO_4$ 0.2 gr. Distilled water 100 c.c.	Slight	Slight	Slight
36	Ammonium tartrate 1 gr. Na_2HPO_4 0.2 gr. Distilled water 100 c.c.	None	None	None

All made neutral to phenolphthalein with NaOH.

These experiments were repeated several times with the same results; a good growth always occurred on media 33 and 34; a visible growth on 35, and no growth at all on 36.

Solid culture media were then made with the above solutions in the same way that the lactose-asparagin agar was prepared. A series of comparative tests was then conducted in the Laboratory of the Rhode Island State Board of Health. Samples of water known to be slightly polluted were plated on the ordinary Standard litmus-lactose agar and at the same time on azolitmin-lactose-asparagin agar, azolitmin-lactose-glycerin agar, azolitmin-lactose-ammonium lactate agar, and azolitmin-lactose-malic acid agar. The results appear in the following table (Table 5).

As will be seen from this table the results were very satisfactory. The red colonies were well developed and characteristic of *B. coli* on the four synthetic media. There were fewer red colonies on the synthetic media than on the Standard medium but the numbers

TABLE 5

		Standard Agar			Asparagin Agar			Glycerin Agar			Ammonium Lactate Agar			Malic Acid Agar		
Plate No..........		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Pawtuxet River	Red colonies	5	2	2	5	0	2	0	1	0	0	2	0	0	0	1
Feb. 26, 1908	Total “	20	18	14	12	8	2	0	2	0	0	4	2	0	0	1
Pawtuxet River	Red “	18	16	15	3	0	0	1	3	0	1	1	2	0	0	0
Mar. 11, 1908	Total “	24	22	20	10	9	13	1	3	0	4	3	6	0	0	0
Pawtuxet River	Red “	10	6	7	3	2	3	2	4	4	1	2	2	2	0	1
Mar. 25, 1908	Total “	14	10	14	5	4	4	2	4	5	1	2	2	2	0	1
Pawtuxet River	Red “	10	15	13	2	3	2	3	3	3	3	3	3	2	0	1
April 8, 1908	Total “	25	24	18	2	4	4	4	14	9	5	8	4	2	0	1
Ten Mile River	Red “	2	2	1	1	1	0	0	2	1	0	0	2	1	0	0
Mar. 2, 1908	Total “	16	18	14	14	21	16	12	14	24	8	20	4	3	0	0
Ten Mile River	Red “	4	3	4	1	2	0	2	1	1	3	0	1	0	0	2
Mar. 18, 1908	Total “	20	14	20	4	4	1	3	3	2	3	1	3	0	0	4

were always fairly proportional. Very few colonies other than red ones appeared on the glycerin, ammonium lactate, and malic acid plates. On the asparagin agar plates there were more colonies other than red ones than on the other synthetic media. On the malic acid agar there were never any other colonies than the red ones, and the red ones were themselves in smaller number. In this connection it is suggested that it might be possible in the case of slightly polluted water to use malic acid agar to plate a larger amount than 1 c.c. of the water to be tested. Experiments were not carried on along this line. No spreading colonies ever appeared on the four synthetic media while there were many on the Standard agar plates. Some of the red colonies on the Standard agar were not at all characteristic of *B. coli* while the red colonies on the four synthetic media were all of the *B. coli* type.

It was then thought advisable to prove whether all the red colonies on the four synthetic media were *B. coli.* All the red colonies were carefully fished from the plates made April 8, 1908, from the Pawtuxet River water. There were 38 red colonies fished from the three Standard agar plates as controls, seven from the three asparagin agar plates, nine from the three glycerin agar plates, nine from the three ammonium lactate agar plates and three from the three malic acid agar plates. Each one was run through the following tests for *B. coli:* (1) morphology; (2) agar slant; (3) gelatin stab; (4) fermentation of dextrose; (5) coagulation of milk; (6) reduction of nitrates.

The results are as follows:

TABLE 6.

Origin of the Colonies	No. of Red Colonies	No. of *B. coli* Colonies	Percentage of *B. coli*
Standard litmus-lactose agar	38	18	48
Litmus-lactose-asparagin agar	7	7	100
Litmus-lactose-glycerin agar	9	9	100
Litmus-lactose-ammonium lactate agar	9	9	100
Litmus-lactose-malic acid agar	3	3	100

The above table shows that of all the red colonies which developed on the Standard litmus-lactose agar only about half of them were *B. coli*, while all the red colonies which developed on the four synthetic culture media were true *B. coli*. The above table also shows that there were more *B. coli* per c.c. of water developing on the ordinary agar than on the synthetic media.

SUMMARY.

1. *B. coli* does not grow on dilute (1 per cent) solutions of asparagin, glycocoll, or urea.

2. It grows very well on a 1 per cent solution of asparagin if 0.2 per cent of sodium phosphate is added.

3. Of all the soluble inorganic salts sodium and ammonium nitrate and sodium and ammonium phosphate are the only ones which can be used with asparagin for the growh of *B. coli*.

4. Substances having an asymmetric carbon atom in their molecule and a CHOH group, such as glycerin, ammonium lactate, malic acid, can be used instead of asparagin for the growth of *B. coli*.

5. These substances seem to favor the growth of *B. coli* and inhibit the growth of water organisms.

6. Very simple synthetic culture media can be used in place of the ordinary litmus-lactose agar or the various bile media and they have several advantages in the routine work of detecting *B. coli* in water.

7. Of the four synthetic media suggested two are especially recommended. Their composition is as follows:

I

Purified agar (3 per cent solution)	500 c.c.
Glycerin 5 gr. Ammonium phosphate 1 gr. Distilled water 500 c.c.	500 c.c.

Sodium hydroxide solution is used to neutralize and 1 per cent lactose is added just before sterilization.

II

Purified agar (3 per cent solution)	500 c.c.
Ammonium lactate 5 gr. Disodium phosphate 1 gr. Distilled water 500 c.c.	500 c.c.

Sodium hydroxide solution is used to neutralize and 1 per cent lactose is added as before.

Both of the above media are to be used with 1 per cent azolitmin solution in the usual way.

8. These media have thus far given extremely satisfactory results for the isolation of *B. coli* in practical water analysis.